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Sustained gene expression in transplanted skin fibroblasts in rats

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Retrovirus-mediated gene transfer into adult skin fibroblasts has provided measurable amounts of therapeutic proteins in animal models. However, the major problem emerging from these experiments was a limited time of vector encoded gene expression once transduced cells were engrafted. We hypothesized that sustained transduced gene expression in quiescent fibroblasts in vivo might be obtained by using a fibronectin (Fn) promoter. Fibronectin plays a key role in cell adhesion, migration and wound healing and is up-regulated in quiescent fibroblasts. Retroviral vectors containing human adenosine deaminase (ADA) cDNA linked to rat fibronectin promoter (LNFnA) or viral LTR promoter (LASN) were compared for their ability to express ADA from transduced primary rat skin fibroblasts in vivo. Skin grafts formed from fibroblasts transduced with LNFnA showed strong human ADA enzyme activity from 1 week to 3 months. In contrast, skin grafts containing LASN-transduced fibroblasts tested positive for human ADA for weeks 1 and 2, were faintly positive at week 3 and showed no human ADA expression at 1, 2 and 3 months. Thus, a fibronectin promoter provided sustained transduced gene expression at high levels for at least 3 months in transplanted rat skin fibroblasts, perhaps permitting the targeting of this tissue for human gene therapy.

Keywords: *gene therapy; retroviral vector; gene transfer; gene inactivation; genes regulatory sequences*

Introduction

Skin fibroblasts can be easily obtained from small biopsies, rapidly grown to large numbers in culture and efficiently transduced with retroviral vectors. More importantly, they can be conveniently implanted as skin equivalent grafts *in vivo*, and the nature of skin as a surface organ enables grafts to be easily monitored and retrieved if necessary. Transduced skin fibroblasts are able to deliver systemically therapeutic proteins, a reflection of their role in secreting cytokines involved in local, as well as systemic, metabolism and immunological function. Because no foreign material is necessary for cell engraftment, as is the case with polytetrafluoroethylene fibers or encapsulation devices, adverse immunological responses to implants are avoided.

Long-term *in vivo* gene expression requires both target cells and gene delivery vectors that permit continuous vector encoded activity. Of the common virus-based methods of gene transfer, retroviral vectors are probably the most useful for *ex vivo* gene transfer.[1–4] For *ex vivo* gene transduction adeno-associated virus vectors have many attractive features, such as safety and ability to transduce non-proliferating cells[5–8] but do not possess advantages over retroviruses. Replication-defective retroviral vectors can be made with high titers, will infect a wide variety of cell types and infection results in stable proviral integration into the host chromosome providing gene expression for the lifetime of the cell and its progeny.[1–4] Recently, the incorporation of internal ribosome entry sites from picornaviruses into retroviral vectors has allowed the generation of bicistronic vectors and subsequent advantages in linked-gene selection.[9–11]

Non-hematopoietic cells other than fibroblasts studied as vehicles for gene therapy include myoblasts, vascular smooth muscle cells and keratinocytes. Transduced skeletal myoblasts have been used to deliver erythropoietin in mice[12–14] and transplantation of retrovirally transduced skeletal muscle myoblasts has been successfully achieved in dogs with alpha-L-iduronidase deficiency.[15] Smooth muscle cells are present within the vasculature as a multilayered mass of long-lived cells in proximity to the circulation and have been investigated as targets for gene therapy.[16–22] *In vivo* gene expression from transduced keratinocyte implants has also been studied.[23–25]

Retrovirus-mediated gene transfer into primary skin fibroblasts has provided measurable amounts of therapeutic proteins in animal models, including adenosine deaminase (ADA),[26,27] factor IX,[28,29] β-glucuronidase[30,31] and erythropoietin.[32] A major problem emerging from experiments using skin equivalent grafts was the limited time of vector-encoded gene expression once transduced cells were implanted *in vivo*. We have shown in both rats and dogs that vector-derived gene expression was diminished at between 3 and 4 weeks despite the long-term presence of vector sequences in transplanted cells.[26,27] In transplanted keratinocytes a similar property of down-regulation of vector gene expression after cell implantation has been reported.[33] However, we showed that the same promoters in retroviral vector constructs permitted stable gene expression for several months in transduced

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hematopoietic stem cells[34,35] and smooth muscle cells.[16,18,22] These data suggested tissue-specific vector inactivation. When fibroblasts are in a quiescent state the choice of promoter for the transduced gene was a major determinant for long-term *in vivo* gene expression.[36,37] When dihydrofolate reductase (DHFR) and CMV promoters were compared, long-term expression of β-galactosidase was only achieved by DHFR promoter.[36] This difference may reflect inactivation of the CMV viral promoter whereas DHFR, a house-keeping promoter, can still be active in quiescent cells. However, these studies used mouse embryo fibroblasts which may not correlate well with gene expression in non-embryonic cells, the only cells available for gene therapy protocols. Vector inactivation could be the reason, at least in part, why viral promoters, such as SV40, Moloney LTR as well as CMV immediate-early promoter drive high levels of gene expression in cultured skin fibroblasts, but became inactive after cells were implanted *in vivo*.[26–28,38] Down-regulation of retroviral vector sequences in transplanted keratinocytes has also been reported.[33] In mice, phosphoglycerate kinase promoter has been used to derive long-term expression of β-glucuronidase[30,38] and erythropoietin[32] in retrovirally transduced fibroblasts implanted into 'neo-organs' in the peritoneal cavity. The applicability of these data to transduced fibroblasts implanted in skin equivalent grafts is not known.

Fibronectin is a widely distributed extracellular matrix protein that plays a key role in cell adhesion, migration and wound healing.[39] The fibronectin gene encodes a 250 kDa dimeric glycoprotein and although produced in many cell types the two major isoforms are synthesized by fibroblasts and hepatocytes. We hypothesized that sustained gene expression in quiescent fibroblasts might be obtained from a fibronectin promoter. Of particular importance to our proposed use, fibronectin is up-regulated in quiescent/senescent fibroblasts.[40,41] The fibronectin promoter from humans and rats has been well characterized and regulatory elements identified.[42,43] We were encouraged by data showing that fibronectin increased and reached high levels in cells which were irreversibly arrested in G_0 phase and which had apparently exhausted their finite division potential.[40] Additionally, a construct encoding a fibronectin promoter achieved high level expression of human interferon γ in quiescent rat 3Y1 cells.[44] When administered to mouse brain to correct lysosomal storage disease, retroviral vector transduced fibroblasts employing a viral promoter appeared to be down-regulated after implantation.[45] Of interest, this study reported that the fibronectin matrix intercalating the implanted cells was present at least 1 year after cell implantation, suggesting that the fibronectin promoter, unlike the vector promoter, was not inactivated.[45] Therefore, to achieve long-term gene expression in fibroblasts *in vivo* we studied retroviral vectors encoding a rat fibronectin promoter.[42]

Results

Expression of human ADA in cultured fibroblasts

LNFnA, the viral vector expressing human ADA form the Fn promoter had a titer of 1×10^7 c.f.u./ml. which was similar to the titers obtained from LASN and LNSA (Figure 1). The retroviral vectors encoding human ADA were tested for transduced gene expression in primary fibroblasts obtained from both a patient with ADA deficiency and Fisher 344 rats. Pooled populations of G418-resistant cells were assayed for total ADA activity (Table 1). All the vectors tested showed significant expression of human ADA in enzyme-deficient patient fibroblasts. The viral LTR promoter produced the highest level of human ADA at activities comparable to our previous findings.[27] Although the promoters derived from simian virus 40 and fibronectin were of similar activity in ADA-deficient human fibroblasts, the Fn promoter was the strongest. Total ADA-activities in transduced rat fibroblasts were of the same order as patient fibroblasts, indicating similar promoter activities in both human and rat cells. In rat fibroblasts the viral LTR promoter was about five-fold more active than the other promoters tested (Table 1). These results showed that, although the Fn promoter was three- to five-fold weaker than MoMLV LTR, it was as strong as simian virus 40, a promoter shown to be of high activity *in vivo*.[35]

In vivo gene expression from transduced rat skin fibroblasts

Initial experiments monitored gene expression for up to 5 weeks because in previous experiments vector inactivation had occurred by this time.[27] Test rats received two skin implants each, one constructed with fibroblasts transduced with LNFnA vector and the other with LASN transduced cells and were harvested at intervals of 1 to 5 weeks (Figure 2). Skin grafts formed from fibroblasts transduced with the vector encoding fibronectin promoter to drive human ADA (LNFnA) showed strong human enzyme activity from week 1 to week 5. Skin grafts containing LASN-transduced fibroblasts, where human ADA is driven from the viral LTR promoter, tested positive for human ADA for weeks 1 and 2, were faintly positive at week 3 and showed no human ADA expression at week 4 (Figure 2, left side). The inactivation of LASN-transduced fibroblasts *in vivo* confirms our previous studies.[26,27] In a second series of experiments, rats received implants which were harvested at intervals of 1, 2 and 3 months. Skin grafts formed from fibroblasts transduced with the vector encoding fibronectin promoter showed strong human enzyme activity at 1, 2 and 3 months (Figure 2, right panel). Some variability of observed human ADA expression may be due in part due to difficulty of harvesting the collagen implant alone and not including adjacent areas of non-transduced cells, particularly at time points beyond 1 month. At these times wound healing has occurred and engrafted tissue was not easily distinguishable from graft bed. For these reasons the specific ADA activities in cell extracts obtained from skin biopsies were variable (data not shown). We attempted to overcome this by inserting four steel clips at the circumference of the graft as an aid to graft retrieval. Skin grafts containing LASN-transduced fibroblasts, where human ADA is driven from the viral LTR promoter, were negative at 1 to 3 months (Figure 2, right panel). These data demonstrate the ability of the fibronectin promoter to provide sustained vector-encoded expression *in vivo*.

Discussion

These studies show that elements of the fibronectin promoter permit sustained transduced gene expression in

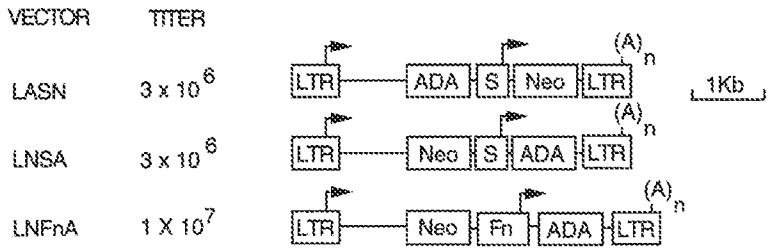

Figure 1 Human ADA vectors. Retroviral vectors LASN, LNSA and LNFnA use three different promoters to express human ADA cDNA: the Moloney murine leukemia virus (MoMLV) promoter in the long terminal repeat (LTR), the simian virus 40 early promoter (S) and the human fibronectin promoter (Fn). Arrows indicate transcription initiation sites and $(A)_n$ are polyadenylation sites. Titers are colony-forming units per milliliter of virus supernate.

Table 1 ADA activity in transduced primary fibroblasts

Virus	*Promoter*	*Fibroblasts*	
		ADA-HDF	*Rat*
None	Native	<0.01	0.9
LASN	LTR	37.7	33.9
LNSA	SV40	8.0	6.6
LNFnA	Fn	11.4	7.1

Enzyme activity units are μmol/h/mg protein. hADA- are human ADA-deficient fibroblasts. Normal human fibroblast ADA activity is 0.9 μmol/h/mg protein.[25] Results are mean of duplicate assays.

transplanted skin fibroblasts. At 1, 2 and 3 months after implantation as skin equivalent grafts, human ADA expression was detectable at levels similar to that observed at 3 weeks, indicating that retroviral promoter inactivation was overcome in the construct encoding Fn promoter. In contrast fibroblasts transduced with LASN vector did not express human ADA beyond 3 weeks, confirming our previous results of vector suppression from skin grafts in both rats[27] and dogs.[26] The critical timepoint in these experiments occurs between 2 and 3 weeks when wound healing has taken place, as judged by the decline of scar tissue and hair regrowth, and implanted fibroblasts become quiescent. The down-regulation of fibroblast gene expression involved in cell proliferation and migration occurs at this time and this process may also down-regulate expression from integrated proviruses. This must be a global event because the transduced fibroblasts are composed of random pooled clones representing multiple integration sites.[27] Methylation of down-regulated proviral sequences has been reported in murine hematopoietic stem cells *in vivo*.[46] However, this may be secondary to vector inactivation and not the primary cause. As the LNFnA vector provided gene expression well beyond the 3- to 4-week time of wound healing previously associated with vector inactivation,[26,27] we anticipate that sustained vector-encoded gene expression will last longer than the 3 months we have demonstrated.

The expression of bacterial neomycin phosphotransferase and human ADA from LNFnA virus did not appear to cause an immune-mediated loss of transplanted cells. This is supported by the sustained human ADA expression from fibroblast implants. The elimination of autologous transduced cells by an immune-mediated mechanism to foreign transgenes has been reported in rats receiving glioma cells[47] and human T cell transplantations.[48] However, the fibroblasts we targeted for gene expression and implantation are not usually involved in antigen processing and presentation and furthermore the

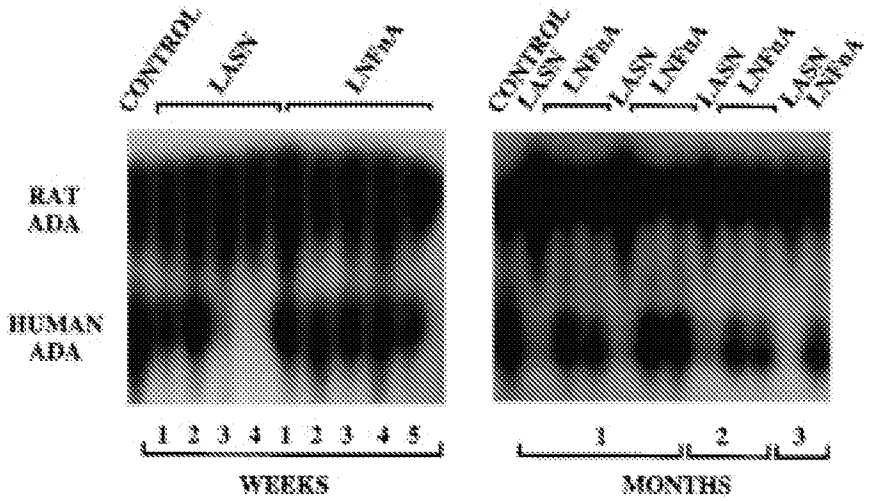

Figure 2 Detection of human ADA in implanted skin fibroblasts. Skin biopsies were harvested at the indicated times and analyzed by starch gel electrophoresis. Rat and human ADA controls were from thymocytes and T lymphoblasts, respectively.

neo and ADA genes are expressed in the cytosol and not secreted.

LNFnA-transduced ADA-negative fibroblasts expressed ADA at 12-fold higher levels than normal fibroblasts, which have an ADA activity of 0.9 μmol per hour per milligram of cell protein.[49] We have previously estimated the number of LNSA-transduced patient fibroblasts necessary to provide treatment at 4×10^8, based on data from red cell therapy.[27] As LNFnA and LNSA vectors express similar amounts of ADA in transduced fibroblasts, then 4×10^8 cells expressing ADA from the fibronectin promoter would provide enzyme to metabolize the cytotoxic ADA substrates deoxyadenosine and adenosine that accumulate in ADA deficiency.[49,50] Transfer of this number of cells is feasible using skin equivalent grafts. It is likely that the calculated number is an overestimate as genetically modified cells continually produce ADA, whereas transfused red cells have a 20- to 30-day half-life with diminishing therapeutic ADA activity. Attachment of a leader sequence to ADA cDNA will allow delivery of ADA to plasma. This should provide increased turnover of cytotoxic ADA substrates because their transportation to and diffusion into the skin graft for metabolism, probably rate-limiting steps, would be eliminated.

The demonstration that the fibronectin promoter provides sustained ADA gene expression enables consideration of treatment of other diseases. It has been demonstrated that fibroblasts can be engineered to secrete enzymatically active clotting factor IX but vector inactivation/suppression in transplanted cells precluded this approach to the treatment of hemophilia.[29] Vectors employing fibronectin promoter will enable this form of treatment of hemophiliacs to be considered. The expression from fibroblast implants of therapeutic cytokines such as erythropoietin for the treatment of anemia associated with end-stage renal disease or granulocyte colony-stimulating factor for neutrophilias may be achievable. The fibroblast-mediated delivery of hormones or cytokines is particularly attractive because, in comparison to enzyme delivery, relatively small amounts of bioactive peptide are required for therapy.

Materials and methods

Cell culture

Primary skin fibroblasts were cultured from skin biopsies obtained from an ADA-deficient patient or Fischer 344 rats.[27] Primary skin fibroblasts, PE501 and PA317 packaging cells and NIH 3T3 TK$^-$ murine fibroblasts were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum at 37°C in an atmosphere containing 5% CO_2 and 1% penicillin and streptomycin. Medium for cell infection contained 4 μg/ml polybrene.

Retroviral vectors

LASN[51] was constructed from LPNSN-2[52] by insertion of human ADA cDNA in place of purine nucleoside phosphorylase (PN) cDNA. LNSA was generated by inserting a 1.2 kb *Cla*I fragment of ADA coding sequences from LNCA[51] into *Cla*I site of plasmid LNSX.[53] LNFnA was constructed by cloning the fibronectin promoter and its 5′ regulatory region *Sma*I + *Pst*I fragment (bp 687 to bp 1426) from plasmid p-RP1 (a kind gift of Dr Richard Hynes, Center for Cancer Research, MIT) into *Nru*I + *Stu*I sites in LNSA. Plasmids containing the viral construct were transfected into PE501 cells and after 2 days virus was harvested and used to infect PA317 amphotropic producer cells.[53] These cells were selected in medium containing 1 mg/ml G418 and clonal cell lines containing single integrated proviruses were isolated. Virus production from the clonal lines was measured by using NIH 3T3 TK$^-$ cells as recipients.[52]

Implantation of transduced fibroblasts

Primary rat skin fibroblasts were implanted in collagen matrices.[26] Briefly, infected and G418-selected fibroblasts are treated with trypsin, washed and for each patch 1×10^6 cells were resuspended in 2 ml of medium, 2 ml of collagen solution in 0.1 M HCl (3 mg/ml rat collagen type I, tissue culture grade, Sigma), 2 ml of 0.1 M NaOH to neutralize the acidic collagen solution, 2 ml 2 × DME and 2 ml rat serum. The mixtures were plated in 10-cm bacterial dishes and formed condensed matrices after 3 days in culture (final size about 2 cm diameter × 1 mm thick). Two collagen–fibroblast matrices were implanted subcutaneously per rat, one on each flank and four steel clips were placed equidistantly around each graft. We found this necessary because otherwise at time-points beyond 1 month, after which wound healing had occurred, the grafts were difficult to locate. At time-points from 1 week to 3 months implants were removed for ADA assay.

ADA assays

Skin graft biopsies were macerated and cultured cells pelleted, sonicated on ice, centrifuged and clear supernate assayed for ADA activity by spectrophotometric assay with adenosine as substrate.[35] Human ADA expression in skin graft extracts was assessed by using starch gel electrophoresis to separate human from endogenous rat ADA.[34,35]

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